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(54) High estrogen-sensitive medaka fish

(57) (1) Transgenic medaka fish into which a polynucleotide having the nucleotide sequence from 211 to 1935 position represented by Sequence ID No: 1 is introduced, (2) A method of producing medaka fish having one or more thrombi, comprising the step of raising the transgenic medaka fish described in (1) in the presence of estrogen, (3) Medaka fish having one or more thrombi produced by the method described in (2), and (4) A method of testing an estrogen-like acting substance, comprising the steps of raising the transgenic medaka fish described in (1) in test water, and observing whether or not one or more thrombi are formed in the medaka fish after the raising step.

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Description

[0001] The present invention relates to high estrogen-sensitive medaka fish, and more specifically, to transgenic medaka fish having a medaka-derived estrogen receptor gene introduced.

[0002] The transgenic medaka fish of the present invention can be used to detect an estrogen-like endocrine disrupting chemicals, and also used as an experimental animal for elucidating development mechanism of thrombosis and as bioassay system for developing a therapeutic agent of thrombosis.

[0003] Recently, the effects of chemical substances present in the environment upon endocrine system of an organism have been intensively studied, and have attracted a high interest increasingly. Since many chemical substances having endocrine disrupting activity upon an organism exhibit estrogen-like action, most of studies are directed to estrogen which is a female hormone.

[0004] Pollution of rivers with such estrogen-like chemical substances has constituted a social problem on a global scale. To detect the estrogen-like chemical substances from a river in the present invention, we tried the use of medaka fish which has excellent features as an experimental animal. However, the medaka fish cannot be used as an aquatic animal for testing environmental water, since it is not sensitive even to an extremely small amount of estrogen. This problem was found for the first time by the present inventor in the course of making the present invention.

[0005] The present invention was made to overcome the aforementioned problem. An object of the present invention is to provide transgenic medaka fish having sensitivity to a very small amount of estrogen. Another object of the present invention is to provide a method of producing medaka fish having one or more thrombi by using the transgenic medaka fish, and to provide the medaka fish having one or more thrombi produced by the method. A further object of the present invention is to provide a method of testing an estrogen-like acting substance by using the transgenic medaka fish.

[0006] To attain the aforementioned objects, the present inventor have succeeded in preparing transgenic medaka fish having a medaka-derived estrogen receptor gene introduced, namely, high estrogen-sensitive medaka fish. The present invention was made based upon such achievement.

[0007] To be more specific, the present invention was achieved by the means described below.

(1) A polynucleotide having a nucleotide sequence represented by Sequence ID No: 1.
(2) A polynucleotide comprising the nucleotide sequence from 211 to 1935 position represented by Sequence ID No: 1.
(3) A protein having an amino acid sequence encoded by the polynucleotide described in (2).
(4) A recombinant vector containing the polynucleotide described in (1) or (2).
(5) Transgenic medaka fish into which the polynucleotide described in (1) or (2) is introduced.
(6) A method of producing medaka fish having one or more thrombi, comprising the step of raising the transgenic medaka fish described in (5) in the presence of estrogen.
(7) Medaka fish having one or more thrombi, which is obtained by raising the transgenic medaka fish described in (5) in the presence of estrogen.
(8) A method of testing an estrogen-like action in test water, comprising the steps of:

raising the transgenic medaka fish described in (5) in the test water; and
observing whether or not one or more thrombi are formed in the medaka fish after the raising step.

(9) The method described in (8), wherein the test water is water taken from environment.
(10) The method described in (8), wherein the test water is water having a test substance added.

[0008] This summary of the invention does not necessarily describe all necessary features so that the invention may also be a sub-combination of these described features.

[0009] Now, the present invention will be explained in detail.

[0010] In the present invention, the medaka fish to be used in cloning an estrogen receptor gene and the medaka fish to be used in introducing the cloned gene are not particularly limited, as long as they belong to a species Oryzias latipes. The medaka fish actually used in the present invention were obtained from the BioScience Center, Nagoya University. The obtained medaka fish were grown while feeding Tetramin (Tetra) in an amount of about 1-10 mg/day.

[0011] It should be noted that fertilized eggs of the medaka fish cannot be deposited, because a technology for resuming the development of a freeze-stored egg, as the need arises, has not yet been established. Therefore, the medaka fish used in the present invention are now raised under control of the present inventor with responsibility. As described in the above, the medaka fish to be used in the present invention are not limited to those raised by the present inventor, and any medaka fish may be used in the present invention.

{Cloning of Medaka-derived estrogen receptor gene}

[0012] The medaka-derived estrogen receptor gene of the present invention is cloned from liver cDNA library of adult medaka fish. More specifically, the medaka-derived estrogen receptor cDNA is cloned by preparing a probe based on a nucleotide sequence of a human-derived estrogen receptor gene and screening the above cDNA library by use of the probe.

[0013] The nucleotide sequence of the cloned estrogen receptor cDNA is determined, and the amino acid sequence predicted from the nucleotide sequence is determined. The nucleotide sequence of the medaka-derived estrogen receptor cDNA and the amino acid sequence are shown by Sequence ID No: 1 in the Sequence Listing.

[0014] In the present invention, the nucleotide sequence for expressing medaka estrogen receptor may have arbitrary length, as long as it comprises at least a coding region (i.e., the nucleotide sequence from 211 to 1935 position represented by Sequence ID No: 1). Furthermore, in the present invention, the amino acid sequence of the medaka estrogen receptor may also have deletion, addition and/or substitution of one or several amino acids in the amino acid sequence represented by Sequence ID No: 1, as long as it has the same function as the protein consisting of the amino acid sequence represented by Sequence ID No: 1.

{Preparation of Recombinant vector}

[0015] The medaka-derived estrogen receptor cDNA cloned by the aforementioned method is introduced into a vector. The introduction of the cDNA into a vector can be performed in accordance with a known genetic engineering process. In this manner, it is possible to prepare a recombinant vector into which the medaka-derived estrogen receptor gene is inserted.

[0016] The vector to be used in the present invention is not particularly limited, as long as it can express the protein encoded by a foreign gene inserted therein. In the present invention, it is preferable to use a plasmid having a promoter sequence and a poly (A) signal sequence, as a vector. For example, as described later in examples, a new plasmid vector is constructed by purifying each DNA fragment from another plasmid containing a DNA fragment of a medaka actin promoter and from another plasmid containing a DNA fragment of a SV40 poly (A) signal, and the resultant plasmid vector may be used.

{Preparation of Transgenic medaka fish}

[0017] The recombinant vector prepared as mentioned above is transferred into a nucleus of a medaka fertilized egg, thereby preparing transgenic medaka fish capable of expressing the estrogen receptor gene in an excessive amount. As the medaka fertilized egg to be transformed in the present invention, an embryo at a single-cell stage or a two-cell stage within one hour after fertilization is preferable. The recombinant vector can be transferred into the fertilized egg by means of a known microinjection procedure.

[0018] The fertilized eggs which have been subjected to the gene transfer operation are preferably hatched in a medaka physiological saline solution (7.5 g/L NaCl, 0.2 g/L KCL, 0.2 g/L $CaCl_2$, 0.02 g/L $NaHCO_3$) at 25-28°C. The hatched medaka fish are raised for about 4 months until they become adult fish.

[0019] From the adult fish, the fish actually having the gene introduced are screened by the following method. A DNA is first extracted from the adult fish. Then, from the extracted DNA, the estrogen receptor gene is amplified by a PCR, and the amplified DNA fragment is subjected to electrophoresis. The estrogen receptor gene inherently present in the chromosome of wild medaka fish has intron, whereas the estrogen receptor gene introduced into the medaka fish herein is a cDNA having no intron. Therefore, two types of estrogen receptor genes can be distinguished by electrophoresis on the basis of the difference of the size of DNA bands. By such procedure, it is possible to screen the medaka fish having the gene successfully introduced. However, it is not clear whether or not the estrogen receptor gene is introduced into the chromosome of a germ cell (sperm or egg) of the medaka fish screened in this step.

[0020] Subsequently, the medaka fish having the estrogen receptor gene introduced into the chromosome of a germ cell are screened by the following method. The medaka fish which has been confirmed to have the gene introduced by the aforementioned method is crossed with wild medaka fish, thereby obtaining offspring. If the medaka fish inheriting the estrogen receptor gene introduced is identified among the offspring, its parent turns out to be desired medaka fish having the estrogen receptor gene introduced in the chromosome of a germ cell. Whether or not the medaka offspring inherits the introduced gene can be identified, in the same way, by electrophoresis on the basis of the difference of the size of DNA bands between the introduced gene and the inherent gene.

[0021] The screened medaka fish having the estrogen receptor gene introduced in the chromosome can inherit the gene from generation to generation. Such medaka fish corresponds to a desired transgenic medaka fish of the present invention.

[0022] The expression of the estrogen receptor gene in the transgenic medaka fish of the present invention was

checked by RT-PCR, and it was confirmed that the estrogen receptor gene was actually expressed. On the other hand, the expression of the estrogen receptor gene was rarely detected in wild medaka fish. As described above, the transgenic medaka fish of the present invention has a large number of estrogen receptors by the expression of the introduced gene. For this reason, the transgenic medaka fish of the present invention shows sensitivity even to a very small amount of estrogen.

{Preparation of Medaka fish having one or more thrombi}

[0023] In the present invention, it was found that the medaka fish having one or more thrombi can be prepared, even if the transgenic medaka fish of the present invention is raised in the presence of estrogen whose concentration is lower than that capable of inducing the formation of a thrombus in wild medaka fish.

[0024] As the transgenic medaka fish to be used for the formation of a thrombus, it is preferable to employ an embryo within 12-24 hours after fertilization, because the formation of a thrombus in the embryo can be easily observed under a dissecting microscope. To form a thrombus in the transgenic medaka fish of the present invention, it is sufficient to contain estrogen in the concentration of 10-20 ng/L, preferably 100-200 ng/L, in the environment for raising medaka fish. It is noted that wild medaka fish require estrogen in the concentration of 4 mg/L or more in order to form a thrombus. As the term of raising the medaka fish in the presence of estrogen, a period of three to four days is necessary for the formation of a thrombus. The growth conditions may be the same as those generally employed for raising medaka fish.

[0025] It is possible to confirm the formation of a thrombus under a dissecting microscope.

[0026] The medaka fish having one or more thrombi prepared in this manner is useful for studying therapy of thrombosis.

{Method of testing an Estrogen-like acting substance}

[0027] The presence or absence of the estrogen-like acting substance can be detected by growing the transgenic medaka fish of the present invention in test water to be checked for the estrogen-like action and then observing whether or not a thrombus is formed in the medaka fish.

[0028] As the transgenic medaka fish to be used in order to detect an estrogen-like acting substance, an embryo within 12-24 hours after fertilization is preferable because it is easily observed under a dissecting microscope.

[0029] The "test water" used herein may be either water collected from the environment (river etc.), which may perhaps contain an estrogen-like acting substance, or water added a chemical substance, which may perhaps act like estrogen, as a test substance. When the former water taken from the environment is used, it is possible to detect the presence or absence of the estrogen-like acting substance in the environmental water. When the latter water added a test substance is used, the estrogen-like action of the test substance can be checked.

[0030] When the test substance is added to the test water, it is preferable to set a concentration thereof at 10 ng/L to 1 mg/L. As the period for raising the medaka fish in the test water, 3-6 days are appropriate. The growth conditions may be the same as those usually raising medaka fish.

[0031] It is possible to observe the formation of a thrombus under a dissecting microscope.

[0032] In fact, an estrogen-like acting chemical substance is known to have an endocrine disrupting action even in an extremely small amount. Therefore, the transgenic high estrogen-sensitive medaka fish of the present invention is very useful in both the aforementioned tests of the environmental water and the test substance.

[Examples]

[0033] Now, examples of the present invention will be explained.

Cloning of Medaka-derived estrogen receptor cDNA

1. Construction of liver cDNA library of Medaka

[0034] Total RNA (10 mg) was obtained from 20 livers of female adult medaka fish by use of RNeasy Maxi Kit (QIAGEN #75162) in accordance with the attached protocol. Then, mRNA (100 μg) was isolated from the obtained total RNA by using Oligotex TM-dT30<Super> (TaKaRa w9021B) in accordance with the attached protocol. Using the isolated mRNA, cDNA (about 10 μg) was synthesized by a cDNA Synthesis Kit (STRATAGENE SC200401) in accordance with the attached protocol. The synthesized cDNA (1 μg) was ligated with λ ZAPII (STRATAGENE SC237211) (1 μg) by using Ligation High (STRATAGENE LGK-101) in accordance with the attached protocol. The total amount of the reaction solution is packaged by using Gigapack III Packaging Extracts (STRATAGENE SC200202) in accordance with the attached protocol. In this manner, a cDNA library constituted of about one million phages was prepared.

2. Screening

[0035] Plasmid pOR8 (about 1 μg) having a human-derived estrogen receptor cDNA (Nature, Vol. 320, pages 134-139, 1986) was digested with a restriction enzyme EcoRI, and then the total amount containing the digested fragments was subjected to electrophoresis on 1.0% agarose gel. After the gel was stained with ethidium bromide, a piece of the gel containing an estrogen receptor cDNA fragment (about 2.1 kb) was excised out, while the size of the DNA fragment was checked on a UV transilluminator. From the gel piece, the estrogen receptor cDNA fragment was purified by using Ultrafree-MC (Millipore) in accordance with the attached protocol. Using the purified cDNA fragment as a probe, the aforementioned cDNA library was screened by the method described in "Molecular cloning - a laboratory manual" (Second edition, J. Sambrook, E.F. Fritsch, T. Maniatis, Cold Spring Harbor Laboratory Press, Pages 2,108-2,125, 1989). In this manner, a recombinant phage having a Medaka estrogen receptor cDNA was isolated. From the isolated phage, plasmid pMER having the Medaka estrogen receptor cDNA was obtained by using an Ex-Assist™ helper phage (STRATAGENE SC237211) in accordance with the attached protocol.

3. Determination of Nucleotide Sequence

[0036] The nucleotide sequence was analyzed by a Dye Terminator Cycle Sequencing method using an Applied Biosystems 373A DNA Autosequencer. As a result of the nucleotide sequence analysis, it was found that the obtained cDNA encodes a protein consisting of 620 amino acids, and that the amino acid sequence of the protein has a high homology with those of estrogen receptors of human and other vertebrates. Therefore, it is conceivable that the cDNA encodes Medaka estrogen receptor.

Preparation of Plasmid pOL22 to be injected in Medaka fertilized eggs

[0037] To express the above isolated Medaka estrogen receptor cDNA in a cell of medaka fish, a promoter derived from a medaka β-actin gene was ligated to the upstream of the 5' end of the cDNA, and an SV40 virus-derived poly (A) addition signal was ligated to the downstream of the 3' end of the cDNA. Plasmid pOL22 having such chimera gene was prepared by the following method.

1. Plasmid pOBA-109 (about 1 μg) (Molecular Marine Biology and Biotechnology, Vol. 3, pages 192-199, 1994) having a medaka β-actin promoter was digested with two types of restriction enzymes of SphI (TOYOBO) and PstI (TOYOBO) in accordance with the attached protocol. After the reaction solution was treated at 70°C for 10 minutes, the total amount of the solution was further treated with Klenow polymerase (TOYOBO) (2 μL) in accordance with the attached protocol. Thereafter, the reaction solution was treated at 70°C for 10 minutes, and then the total amount of the solution was subjected to electrophoresis on 1.0% agarose gel. As a result, a gel piece containing about 3.5 kb of a medaka β-actin promoter DNA fragment was excised out. From the excised gel piece, the medaka β-actin promoter DNA fragment was purified by using Ultrafree-MC (Millipore) in accordance with the attached protocol.
2. Plasmid pS65T-C1 (about 1 μg) (Clontech) having the SV40 poly (A) signal was digested with two types of restriction enzymes of AseI (TOYOBO) and NheI (TOYOBO) in accordance with the attached protocol. After the reaction solution was treated at 70°C for 10 minutes, the total amount of the solution was further treated with Klenow polymerase (TOYOBO) (2 μL) in accordance with the attached protocol. Thereafter, the reaction solution was treated at 70°C for 10 minutes, Bacterial alkaline phosphatase (TOYOBO) (2 μL) was added thereto, and the resultant solution was mixed and reacted at 60°C for 2 hours. The total amount of the reaction solution was subjected to electrophoresis on 1.0% agarose gel, and a gel piece containing about 4.1 kb of the SV40 poly (A) signal DNA fragment was excised out. From the gel piece, the SV40 poly (A) signal DNA fragment was purified by using Ultrafree-MC (Millipore) in accordance with the attached protocol.
3. Two DNA fragments purified in the above (5 μL for each) each containing 0.1 μg of DNA were mixed with 10 μL of solution I (TaKaRa) of DNA Ligation System Ver. 2, and the mixture was incubated at 16°C for about 12 hours. In this manner, the two DNA fragments were ligated to each other. Using the resultant reaction solution (10 μL), E. coli DH5α (TaKaRa, #9057) was transformed. From the obtained transformants, a plasmid was isolated by using NucleoBond Plasmid Kit (Clontech #K3001-1). The isolated plasmid was designated as pOL21.
4. Plasmid pOL21 (about 1 μg) was digested with a restriction enzyme SalI (TOYOBO) in accordance with the attached protocol. After the reaction solution was treated at 70°C for 10 minutes, the total amount of the solution was further treated with Klenow polymerase (TOYOBO) (2 μL) in accordance with the attached protocol. Thereafter, the reaction solution was treated at 70°C for 10 minutes, Bacterial alkaline phosphatase (TOYOBO) (2 μL) was added thereto, and the resultant solution was mixed and reacted at 60°C for 2 hours. The total amount of the reaction solution was subjected to electrophoresis on 1.0% agarose gel, and a gel piece containing about 7.1 kb

of a DNA fragment was excised out. From the gel piece, the DNA fragment was purified by using Ultrafree-MC (Millipore) in accordance with the attached protocol.

5. To amplify the medaka estrogen receptor cDNA, a reaction solution (50 μL) for PCR was prepared, in accordance with the attached protocol, by using plasmid pMER (10 ng) as a template; 25 pmole of Primer 1 (5'-TCGGTGACATGTACCCTGAA-3') (Sequence ID No: 2) and 25 pmole of Primer 2 (5'-CTGTGTGCTCAGTCTTGAAG-3') (Sequence ID No: 3); and KOD polymerase (TOYOBO) (1 μL). PCR was performed by repeating 25 cycles of the following program: 98°C for 15 seconds, 65°C for 2 seconds, and 74°C for 30 seconds. After the reaction, the reaction solution containing the PCR product was stored at 4°C. An aliquot (5 μL) of the reaction solution containing the PCR product was subjected to electrophoresis on 1% agarose gel. As a result, it was confirmed that the molecular size of the PCR product is about the same as that of a desired product (1.8 kb). From the remaining reaction solution, the amplified DNA fragment was purified by using SUPREC™-02 (TaKaRa) in accordance with the attached protocol. The total amount of the purified DNA fragment was phosphated with 2 μL of T4 kinase (TOYOBO) in accordance with the attached protocol. After the reaction, the resultant solution was treated at 70°C for 10 minutes.

[0038] The DNA fragments (0.1 μg for each) finally obtained in the above steps 4 and 5 were ligated by using DNA Ligation System Ver.2 (TaKaRa) in accordance with the attached protocol. Using the resultant reaction solution (10 μL), E. coli DH5α (TaKaRa #9057) was transformed. From the obtained transformants, a plasmid was isolated by using NucleoBond Plasmid Kit (Clontech #K3001-1). The isolated plasmid was designated as pOL22.

Method of Preparing Transgenic Medaka Fish

[0039] 1. About 500 of the medaka-fertilized eggs either at a single-cell stage or a two-cell stage were taken within one hour after fertilization, and stored at 6°C until DNA injection. About 100 pL of DNA solution (containing 10 μg of plasmid pOL 22 per 1 mL) was injected into the cytoplasm of the fertilized egg by using a glass tube with a sharp end under a dissecting microscope. Thereafter, the fertilized eggs were divided into groups each consisting of 50 eggs. 50 eggs of each group were incubated at 25°C in 40 mL of medaka physiological saline solution (containing 7.5 g of NaCl, 0.2 g of KCl, 0.2 g of $CaCl_2$, and 0.02 g of $NaHCO_3$ per liter) until they were hatched. As a result, about half of eggs were hatched. The hatched eggs were transferred to a water tank, and raised by feeding with Tetramin (Tetra) for about 4 months until they became adult fish. More specifically, the amount of Tetramin per day was set so as not to leave, and it was fed by dividing into three times in a day. As a result, about 50 medaka fish survived until they became adult fish.

[0040] Half of each caudal fin from the survived adult fish was cut off with scissors. DNA (20 μL) was extracted separately from each of the cut caudal fins by using a DNA extraction kit ISOHAIR (WAKO) in accordance with the attached protocol. A reaction solution (100 μL) for PCR was prepared, in accordance with the attached protocol, by using the extracted DNA (1 μL); two types of primers F1 (5'-CTTCCGTGTGCTCAAACTCA-3' (Sequence ID No: 4)) and R1 (5'-GTAGGAGGTCATAAAGAGGG-3' (Sequence ID No: 5)) (50 pmole for each); and Ex Taq (TaKaRa Ex Taq RR001B) (1 μL). After initial denaturing at 94°C for 2 minutes, PCR was performed by repeating 30 cycles of the following program: 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 90 seconds. Finally, the resultant solution was reacted at 72°C for 6 minutes, and then it was stored at 4°C. An aliquot (10 μL) of the PCR solution containing the PCR product was subjected to electrophoresis on 1% agarose gel. In the case of medaka fish having no chimera gene injected, about 1 kb of DNA band was detected, which was derived from amplification of estrogen receptor gene inherently present in the chromosome of wild medaka fish. In contrast, in the case of medaka fish having a chimera gene injected, a 320 bp of DNA band derived from the chimera gene was detected in addition to the above about 1 kb of band. As a result, eight medaka fish with the chimera gene were obtained.

[0041] 3. Eight medaka fish with the chimera gene were individually crossed with wild medaka fish. From each parent medaka fish, one hundred offspring were raised until they became adult fish. DNA (20 μL) was extracted from each caudal fin of these offspring by use of a DNA extraction kit ISOHAIR (WAKO) in accordance with the attached protocol. A reaction solution (100 μL) for PCR was prepared, in accordance with the attached protocol, by using the extracted DNA (1 μL); two types of primers (5'-CTTCCGTGTGCTCAAACTCA-3') (Sequence ID No: 4) and (5'-GTAGGAGGTCATAAAGAGGG-3') (Sequence ID No: 5) (50 pmole for each); and Ex Taq (TaKaRa Ex Tag RR001B) (1 μL). After initial denaturing at 94°C for 2 minutes, PCR was performed by repeating 30 cycles of the following program: 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 90 seconds. Finally, the resultant solution was reacted at 72°C for 6 minutes, and then it was stored at 4°C. An aliquot (10 μL) of the PCR solution containing the PCR product was subjected to electrophoresis on 1% agarose gel. The offspring medaka fish having a 320 bp of DNA band derived from the chimera gene was identified as transgenic medaka fish. As a result, only two of original eight medaka fish actually transferred the chimera gene into their offspring. Therefore, two strains of transgenic medaka fish (designated as strains A and C) were obtained. The number of the transgenic medaka fish obtained herein was small. However, these transgenic

medaka fish were crossed with wild medaka fish, and thereby more than about 100 transgenic medaka fish have been maintained for each strain. In both strains of the transgenic medaka fish, about half of offspring obtained by crossing with wild medaka fish have the chimera gene. From this, it was found that either one of two homologous chromosomes had the chimera gene.

Expression of Chimera Gene in Transgenic Medaka Fish

[0042] The fact that the strains A and C of the transgenic medaka fish produce a mRNA encoding estrogen receptor in a larger amount than wild medaka fish, was demonstrated by the following method. RNA (30 μL) was extracted from about 30 fertilized eggs which were obtained by crossing the transgenic medaka with wild medaka fish and about 30 fertilized eggs which were obtained by mutual mating between wild medaka fish, by use of an RNeasy Mini Kit (QIAGEN) in accordance with the attached protocol. Then, a reaction solution (50 μL) for RT-PCR was prepared by using the extracted RNA (1 μL) ; three types of primers (50 pmole for each): F1 (Sequence ID No: 4), R1 (Sequence ID No: 5) mentioned above, and R2 (5'-GAGGGACTTTGTTCTTGCAC-3') (Sequence ID No: 6); and Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech #27-9267-01) in accordance with the attached protocol. After performing initial reactions at 42°C for 30 minutes and 95°C for 5 minutes, RT-PCR was performed by repeating the 30 cycles of the following program: 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 90 seconds. After completion of the reaction, the reaction solution was stored at 4°C. An aliquot (10 μL) of the RT-PCR solution was subjected to electrophoresis on 1% agarose gel. Thereafter, DNA on the gel was transferred onto a membrane in accordance with the method described in "Molecular cloning - a laboratory manual" (Second edition, J. Sambrook, E. F. Fritsch, T. Maniatis, Cold Spring Harbor Laboratory Press, Pages 9.31-9.62, 1989). Then, Southern Hybridization was performed by using an EcoRI-SalI fragment (354 bp) of the estrogen receptor cDNA as a probe. A clear band of about 300 bp was detected in the fertilized eggs derived from the strains A and C of the transgenic medaka fish. Since the band from the strain C was nearly twice as dense as that from the strain A, it is conceivable that the strain C of the transgenic medaka fish produce the mRNA encoding estrogen receptor in an larger amount than the strain A of the transgenic medaka fish. However, no band was detected in the fertilized eggs derived from the wild medaka fish. From this fact, it is conceivable that the expression amount of the estrogen receptor mRNA is significantly low in the fertilized eggs from the wild medaka fish.

Formation of Thrombus by Estrogen in Transgenic Medaka Fish

[0043] Medaka physiological saline solutions (40 mL for each) containing 17β-estradiol in an amount of 2 ng/L, 20 ng/L, 200 ng/L, 2 μg/L, 1 mg/L, and 4 mg/L were supplied in culture plates, respectively. Three sets were prepared for each concentration. About 30 fertilized eggs (W) of 12-hours after fertilization which were obtained by mutual mating between wild medaka fish, were placed in the first set of culture plates. About 30 fertilized eggs (A) of 12-hours after fertilization which were obtained by crossing the strain A of the transgenic medaka fish with wild medaka fish, were placed in the second set of culture plates. About 30 fertilized eggs (C) of 12-hours after fertilization which were obtained by crossing the strain C of the transgenic medaka fish with wild medaka fish, were placed in the third set of culture plates. After the culture plates were incubated at 25°C for 3 days, medaka embryos were checked under dissecting microscope with respect to formation of a thrombus.

[0044] In almost 100% of the fertilized eggs (W), one or more thrombi were formed at the concentration of 4 mg/L of 17β-estradiol, but were not observed at the concentration of less than 4 mg/L. 1%, 3%, 8%, 49%, 55%, and 100% of the fertilized eggs (A) caused the formation of the thrombi at the concentrations of 2 ng/L, 20 ng/L, 200 ng/L, 2 μg/L, 1 mg/L, and 4 mg/L of 17β-estradiol, respectively. 19%, 41%, 75%, 73%, 60%, and 100% of the fertilized eggs (C) caused the formation of the thrombi at the same concentrations as mentioned above, respectively. It was therefore demonstrated that the estrogen sensitivities of fertilized eggs (A) and (C) are thousand times and hundred thousand times as high as that of the wild medaka fish, respectively.

{Effect of the present invention}

[0045] As described in the foregoing, the transgenic medaka fish of the present invention is high sensitive to an extremely low amount of estrogen, compared to non-transgenic medaka fish (i.e., wild medaka fish). Therefore, the transgenic medaka fish of the present invention is very useful as a novel aquatic animal whereby a very small amount of estrogen-like acting substance present in the environment can be detected quickly, easily, continuously, and inexpensively. By use of such a novel aquatic animal, it is possible to detect water pollution of rivers with an estrogen-like acting substance which is a social problem.

[0046] Also, if the fertilized eggs of the transgenic medaka fish of the present invention are raised in estrogen-containing water, it is possible to observe the formation of thrombus in a blood vessel. Therefore, the transgenic medaka

fish of the present invention is useful as an experimental animal for elucidating the development mechanism of thrombosis caused by intake of estrogen due to the use of oral contraceptive and hormonotherapy in human. The animals conventionally used in thrombosis studies are rats and rabbits. However, they are expensive as experimental animal. In addition, the formation of a thrombus must be checked by injecting an angiographic agent followed by using a specific device. In contrast, the medaka fish used in the present invention has advantages of inexpensiveness and easiness. More specifically, since its fertilized egg is transparent, the thrombus can be observed easily and continuously under a dissecting microscope. Moreover, it is easy to prepare a sample for its biochemical analysis.

[0047] Furthermore, the transgenic medaka fish of the present invention can be used as a model animal for bioassay system of thrombosis, when a therapeutic agent of thrombosis is developed. Although the therapeutic agent of thrombosis has been developed based on administration by injection up to now, the use of the medaka fish of the present invention makes it possible to screen an oral therapeutic agent of thrombosis on a large scale.

SEQUENCE LISTING

<110> The President of Hiroshima University

<120> High estrogen-sensitive medaka fish.

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<223> Primer for medaka estrogen receptor gene

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Claims

1. A polynucleotide having a nucleotide sequence represented by Sequence ID No: 1.
2. A polynucleotide comprising the nucleotide sequence from 211 to 1935 position represented by Sequence ID No: 1.
3. A protein having an amino acid sequence encoded by the polynucleotide according to claim 2.
4. A recombinant vector containing the polynucleotide according to claim 1 or 2.
5. Transgenic medaka fish into which the polynucleotide according to claim 1 or 2 is introduced.
6. A method of producing medaka fish having one or more thrombi, comprising the step of raising the transgenic medaka fish according to claim 5 in the presence of estrogen.
7. Medaka fish having one or more thrombi, which is obtained by raising the transgenic medaka fish according to claim 5 in the presence of estrogen.
8. A method of testing an estrogen-like action in test water, comprising the steps of:

   raising the transgenic medaka fish according to claim 5 in the test water; and
   observing whether or not one or more thrombi are formed in the medaka fish after the raising step.

9. The method according to claim 8, **characterized in that** the test water is water taken from environment.

10. The method according to claim 8, **characterized in that** the test water is water having a test substance added.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 01 11 5472

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	KAWAHARA T ET AL: "Cloning and expression of genomic and complementary DNAs encoding an estrogen receptor in the medaka fish, Oryzias latipes" ZOOLOGICAL SCIENCE, vol. 17, no. 5, July 2000 (2000-07), pages 643-649, XP001030948	1-4	G01N33/18 A01K67/027 C07K14/46 C12N15/12 A61K49/00
A	* the whole document *	5-10	
X	JP 2000 201688 A (SUMITOMO CHEM CO LTD) 25 July 2000 (2000-07-25) SeqIdNo1-4 -& DATABASE GENESEQ EBI Hinxton U.K.; Accession Number: AAA92175, 5 January 2001 (2001-01-05) SUMIMOTO CHEM CO LTD: "JP2000201688: Oryzias lapites oestrogen receptor encoding DNA SEQ ID NO:4" XP002181483 99.678% identity (99.678% ungapped) in 1863 nt overlap (76-1938:1-1863) with SeqIdNo.1 * abstract * -& DATABASE GENESEQ EBI Hinxton U.K.; Accession Number: AAA92174, 5 January 2001 (2001-01-05) SUMIMOTO CHEM CO LTD: "JP2000201688: Oryzias lapites oestrogen receptor encoding DNA SEQ ID NO:2" XP002181484 99.653% identity (99.653% ungapped) in 1728 nt overlap (211-1938:1-1728) with SeqIdno.1 * abstract *	1-4	

-/--

TECHNICAL FIELDS SEARCHED (Int.Cl.7)
G01N
A01K
C07K
C12N
A61K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	29 October 2001	Lonnoy, O

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 01 11 5472

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	DATABASE SWALL EBI Hinxton U.K.; Accession Number: D28954, 23 April 1994 (1994-04-23) OKADA H ET AL: "Oryzias sp. mRNA for estrogen receptor, complete cds." XP002181485 99.928% identity (99.964% ungapped) in 2764 nt overlap (1-2764:8-2770) with SeqIdNo.1 * abstract *	1-4	
X	-& DATABASE SWALL EBI Hinxton U.K.; Acession Number: P50241, 1 October 1996 (1996-10-01) OKADA H ET AL: "Oryzia latipes Estrogen Receptor" XP002181486 * abstract *	1-4	
A	TAKAGI S ET AL: "AN EFFICIENT EXPRESSION VECTOR FOR TRANSGENIC MEDAKA CONSTRUCTION" MOLECULAR MARINE BIOLOGY AND BIOTECHNOLOGY, XX, XX, vol. 3, no. 4, 1 August 1994 (1994-08-01), pages 192-199, XP000199320 * figures 2,4 *	5-10	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
A	GRAY M ET AL: "Reproductive success and behavior of Japanese medaka (Oryzias latipes) exposed to 4-tert-octylphenol" ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY, vol. 18, no. 11, November 1999 (1999-11), pages 2587-2594, XP001030944		
A	US 5 903 305 A (YAMAMOTO TAKAHIRO) 11 May 1999 (1999-05-11)		
	-/--		

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	29 October 2001	Lonnoy, O

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EPO FORM 1503 03.82 (P04C01)

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 01 11 5472

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
P,A	WO 00 49150 A (HE JIANGYAN ;JU BENSHENG (SG); LAM TOONG JIN (SG); YAN TIE (SG); G) 24 August 2000 (2000-08-24)		
P,A	PATENT ABSTRACTS OF JAPAN vol. 2000, no. 22, 9 March 2001 (2001-03-09) & JP 2001 122899 A (SUMITOMO CHEM CO LTD), 8 May 2001 (2001-05-08) * abstract *		
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	29 October 2001	Lonnoy, O

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ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 01 11 5472

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29-10-2001

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 2000201688 A	25-07-2000	NONE	
US 5903305 A	11-05-1999	JP 9229924 A	05-09-1997
WO 0049150 A	24-08-2000	WO 0049150 A1	24-08-2000
JP 2001122899 A	08-05-2001	NONE	

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82